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Histopathological characteristics of a densovirus-like from silkworm, *Bombyx mori*

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Abstract: A small icosahedral nonenveloped DNA virus with the main biological and biophysical properties of densoviruses (DNVs) was isolated from silkworm *Bombyx mori* with abnormal larval mortality. Histopathological studies suggested that the virus first parasited on columnar cell of midgut, and then the nucleus of columnar cell became extremely inflated, or even broken. *In situ* hybridization results also demonstrated the virus was first reproduced in nucleus of columnar cells among midgut epithelial cells. It can also infect and reproduce in the goblet cells and most of the larval tissues at the last stage of infected silkworm.

Key words: *Densovirus*; *Bombyx mori*; histopathological characteristics

1 INTRODUCTION

Densoviruses (DNVs) were among the first parvoviruses discovered in 1964 from *Galleria mellonella* (Meynadier *et al.*, 1964) and in 1968 from *Bombyx mori* (Shimizu, 1975). DNVs often present in a restricted host range and are different from other vertebrate parvoviruses, usually fatal to their hosts. The initial symptoms are anorexia and lethargy, followed by flaccidity and lucency of body (Kawase *et al.*, 1990). Due to the significance of the silkworm industry, many DNVs isolates were obtained from *Bombyx mori* Ina isolate (*Bm*DNV-Ⅰ) isolated from silkworm in Japan belongs to *Iteravirus* genus possessing a genome of 5 kb separately encapsidated in stoichiometric proportion as "plus" and "minus" strands (Shimizu, 1975; Li *et al.*, 2001), whereas Saku isolate (Shimizu and Watanabe, 1984) and Yamanishi isolate (*Bm*DNV-Ⅱ) (Seki and Iwashita, 1983) isolated from silkworm in Japan, and probably Zhenjiang isolate isolated from silkworm in China (Iwashita and Chun, 1982) and the Kenchu isolate (Shyamala *et al.*, 1987) isolated from silkworm in India, belong to the *Bidensovirus* possessing two genomes of 6.1 and 6.6 kb (Tijssen and Bergoin, 1995). *Bm*DNVs reproduce only in nuclei of columnar cells of midgut epithelium. DNVs from *Bombyx*, *Sibine*, *Euxoa* and *Periplaneta* infect predominantly midgut cells. This is strikingly in contrast to *Gm*DNV and DNVs from *Junonia*, *Aedes*, *Agraulis*, *Diatraea*, *Casphalia* and *Pseudoplusia* which infect, almost indiscriminately, all tissues except the midgut (Garzon and Kurstak, 1976). We have isolated a small icosahedral nonenveloped DNA virus with the main biological and biophysical properties of densoviruses from silkworm in Chongqing, China. The virus possesses the genome of 6.5 kb and has no serological reaction with *Bm*DNV-Ⅰ and *Bm*DNV-Ⅱ virion rabbit antibodies. We report here the histopathological characteristics of the *Bm*DNV-like isolated from silkworm.

2 MATERIALS AND METHODS

2.1 Serological examination of virus

Serological examination of virus was conducted with dot blot hybridization, with reference to the method of Franc *et al.* (1991). Antibodies against purified *Bm*DNV-Ⅰ, *Bm*DNV-Ⅱ and *Bm*DNV-like particles were raised by injecting subcutaneously a rabbit with a virus suspension containing 100 mg of viral antigens emulsified in an equal volume of Freund's complete adjuvant, followed by two boosters at 2 weeks' intervals with Freund's incomplete adjuvant. The rabbit was bled before the first injection and two weeks after the third injection.

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Virus suspensions of the three viruses (*Bm*DNV-Ⅰ, *Bm*DNV-Ⅱ and *Bm*DNV-like) with the same concentration (same optical density unit at 260 nm) were subject to five-fold dilution and 2 μL of each dilution was spotted onto one nitrocellulose sheet. The membrane was incubated with the antisera against the three viruses, and following treatment with a second antibody conjugated with peroxidase, the membrane was incubated in the presence of the appropriate chromogenic substrate (0.4% 3-amino-9-ethylcarbazole in dimethylformamide diluted at 1:20 in 0.05 mol/L acetate buffer) containing 0.05% H_2O_2.

2.2　Isolation and characterization of viral nucleic acid

DNA was extracted from virions with a speedy method (Pan *et al.*, 2003). The DNA was precipitated with ethanol from the combined suspensions, resuspended in TE [10 mmol/L Tris-HCl (pH 8.0), plus 1 mmol/L EDTA], and stored at −20°C. Viral nucleic acid type of the new virus was identified by RNase and DNase digested separately, then checked with gel electrophoresis.

2.3　Observation on the cell infected by *Bm*DNV-like through hematoxylin-eosin (HE) staining

After infected by the *Bm*DNV-like isolated from silkworm, 3 −4 silkworms were fixed every 24 h with modified Bouin's Fixative (saturated picric acid: formaldehyde: glacial acetic acid = 75:25:5) for 2 h, then embed with paraffin wax, and sliced. The slices were dewaxed, stained with HE, enveloped with neutral resin, observed and photographed under an Olympus fluorescence microscope(BX51)

2.4　Fluorescence *in situ* hybridization of diseased tissues of infected silkworm

DNV genome labelled with nick translation was prepared. The paraffin slices of infected silkworm were dewaxed, fluorescence *in situ* hybridization was conducted with the instruction of DIG DNA Labeling and Detection Kit by Roche, and photographed under an Olympus fluorescence microscope (BX51).

2.5　Observation of ultramicrostructure of infected tissues

Midgut tissues of silkworm infected by the *Bm*DNV-like were rinsed with sterilized water for several times, fixed with 3% glutaraldehyde and then with 1% osmium tetraoxide solution, dehydrated gradually with acetone according to the regular method, permeated gradually with acetone plus E-pon812, and conducted *in situ* embedding with E-pon812 epoxy resin. The tissues were sliced with a LKB ultra-thin slicer, and stained with uranyl acetate-plumbum citrate. Ultramicrostructure changes of tissues were observed under an electron microscope (H-600A-2).

3　RESULTS

3.1　Serological relationship between *Bm*DNV-like and *Bm*DNV-Ⅰ or *Bm*DNV-Ⅱ

After *Bm*DNV-Ⅰ, *Bm*DNV-Ⅱ, and *Bm*DNV-like hybrided with their respective antibodies by using the immune dot blot hybridization, hybridization signals were produced; however, no hybridization signals were produced after *Bm*DNV-like hybrided with the antibodies of *Bm*DNV-Ⅰ or *Bm*DNV-Ⅱ (Fig. 1), indicating that there was no serological relationship between *Bm*DNV-like and *Bm*DNV-Ⅰ or *Bm*DNV-Ⅱ.

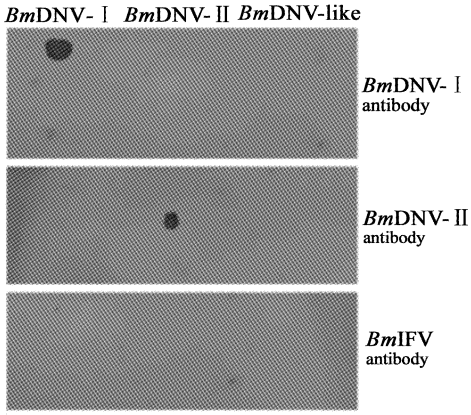

Fig. 1　Immuno dot-blot detection of *Bm*DNV-Ⅰ, *Bm*DNV-Ⅱ and *Bm*DNV-like

The membranes were incubated first with anti-*Bm*DNV-Ⅰ, anti-*Bm*DNV-Ⅱ and *Bm*DNV-like rabbit antibodies, then with an anti-rabbit or mouse IgG antibody coupled to peroxydase. The dot blots were revealed by appropriate chromogenic substrate.

3.2　Features of viral nucleic acid

The agarose electrophoresis of *Bm*DNV-Ⅰ, *Bm*DNV-Ⅱ, and *Bm*DNV-like nucleic acids demonstrated that, the nucleic acid of *Bm*DNV-Ⅰ was 5 kb in length, the electrophoresis of *Bm*DNV-Ⅱ nucleic acid produced two bands, approximately 6.1 and 6.5 kb, respectively, while the nucleic acid of *Bm*DNV-like was 6.5 kb in length, different from those of *Bm*DNV-Ⅰ or *Bm*DNV-Ⅱ (Fig. 2).

After *Bm*DNV-like DNA was treated with DNase and RNase respectively, the electrophoretic test showed that *Bm*DNV-like nucleic acid could be digested by DNase other than RNase (Fig. 3), indicating that the nucleic acid type of *Bm*DNV-like was a DNA virus.

3.3　Characteristics of the cells infected by DNV-like

At 24 h after infection by *Bm*DNV-like , no

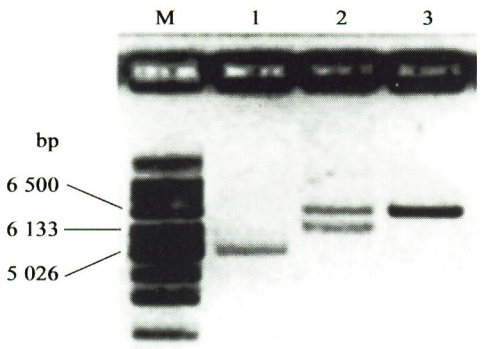

Fig. 2 Nucleic acid size of *Bm*DNV-like

M: DNA molecular weight marker; 1: *Bm*DNV-Ⅰ; 2: *Bm*DNV-Ⅱ; 3: *Bm*DNV-like.

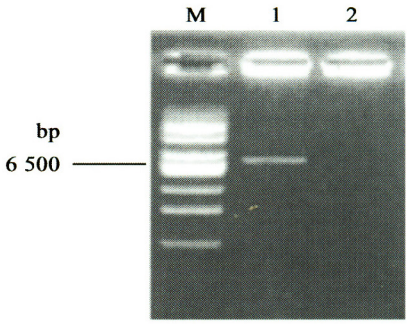

Fig. 3 Nucleic acid of *Bm*DNV-like digested with DNase and RNase

M: DNA molecular weight marker; 1: RNase digestion; 2: DNase digestion.

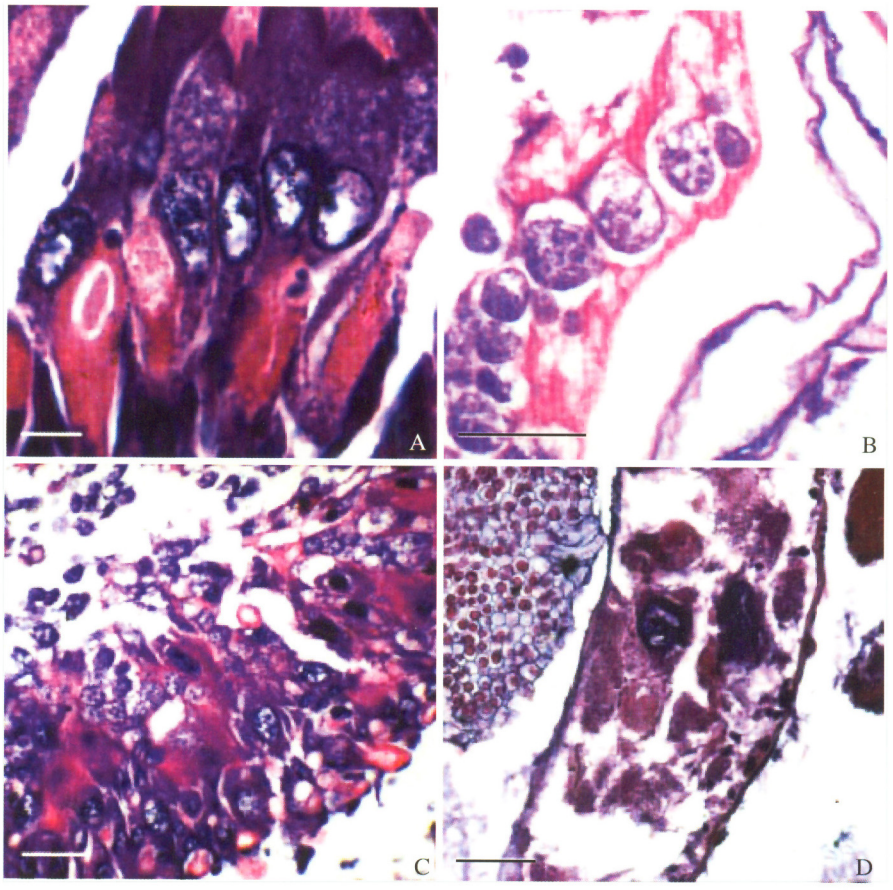

Fig. 4 Characteristics of the cells infected by *Bm*DNV-like

A: Normal cells (nuclei not inflated, bar is 10 μm); B: Nuclei infected is inflated (72 h, bar is 40 μm); C: Infected cells is broken, nuclei is inflated and released (96 h, bar is 45 μm); D: At the last stage of infection, midgut cells are completely destroyed(bar is 30 μm).

obvious inflation was seen in nucleus (Fig. 4: A). At 72 h, the infected nuclei was inflated (Fig. 4: B). At 96 h after infection, a number of infected cells were broken, and the nuclei entered into midgut lumen (Fig. 4: C). At the last stage of infection, all cells of midgut tissue were infected, cells were broken and dropped in gut lumen, and only a layer of basilar membrane were left (Fig. 4: D). At this time, silkworm body was softened and died soon.

Through comparison of the diameter of midgut columnar cells between the virus-infected group and the virus-free group (control), it was found that the difference of nucleus diameters of midgut columnar cells was not obvious on Day 1; however, the differences were gradually enlarged since Day 2, reached maximum on Days 4 and 5, and then gradually decreased later. This was possibly because a number of the inflated

nuclei dropped off. When nuclei were inflated most severely, the average nuclei diameter was 2.64 μm, 2.6 times that of the control group.

3.4 Proliferation characteristics of DNV

At the initial stage infected by *Bm*DNV-like, chromatin of nuclei was homogenous; however, changes occurred in nucleolus, and many nucleoli were produced; those nucleoli developed continuously, and connected finally with each other, forming virus-producing matrix including immature virions (Fig. 5: A). Virions became mature in the virus-producing matrix and were seen in groups in nuclei at the beginning (Fig. 5: B). Along with increased mature virions, they were released into nuclei from the matrix, and distributed extensively in nuclei. *Bm*DNV-like virions were of sphericity, with diameter approximately 22 nm (Fig. 5: C). During the development of virus, we also observed that mitochondrion were inflated and became vacuole, and micro-villi of midgut cells were decreased (Fig. 5: D).

3.5 Site and tissue to be infected by *Bm*DNV-like

The results of FISH showed a pathological course consistent with HE staining. At the early stage of infection by the virus, a few positive signals were found in midgut nuclei. In severe infection by virus, positive signals occurred not only in all midgut cells (Fig. 6: A), but also in other histiocytes including fat bodies (Fig. 6: B). The results of FIHS further demonstrated that, the infected nuclei were inflated because virions proliferated in nuclei , and also showed that, at the last stage, the *Bm*DNV-like could infect midgut round cells and other histiocytes including goblet cells, fat bodies, muscles, and dermal tissues, *etc.*, indicating that this virus caused systemic infection of silkworm (Fig. 6: C).

4 DISCUSSION

Presently, nearly 30 DNVs have been found, but their molecular researches are still less, and their classifications were not detailed. Especially, more than ten strains of *Iteravirus* genus (*Bm*DNV-Ⅰ as a representative) have been found. Considering the disparities size among their genome, Bergoin and Tijssen (2000) suggested that *Bm*DNV-Ⅱ may be isolated from the *Iteravirus* genus and become a new genus – *Bidensovirus*.

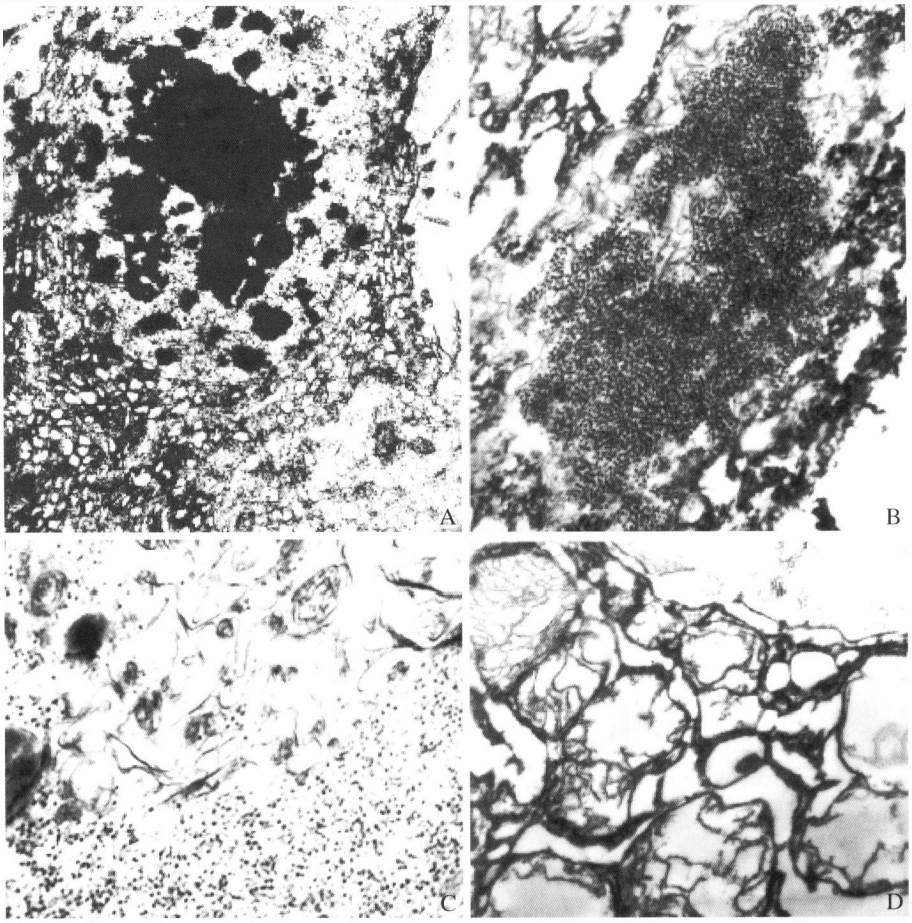

Fig. 5　Proliferation characteristics of *Bm*DNV-like

A: Many nucleoli connected finally with each other, forming virus-producing matrix (10 K); B: Virions in groups in nuclei (25 K); C: Numerous viral particles are visible emerging in whole nuclei (30 K); D: Mitochondrion inflated and became vacuole (17 K).

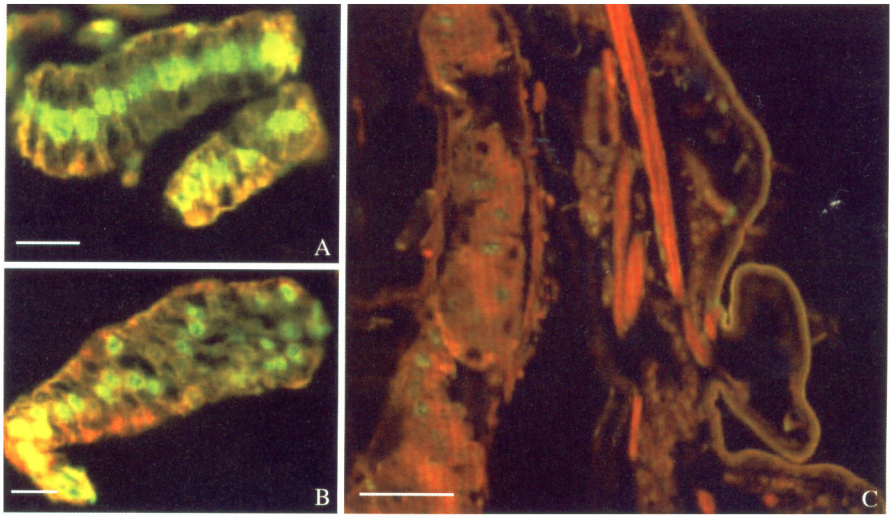

Fig. 6 Sites and tissues infected by *Bm*DNV-like

A: Midgut tissue of silkworm infected by virus; positive signals occur in all midgut cells (bar is 10 μm); B: Positive signals occur in fat bodies of silkworm infected by virus (bar is 10 μm); C: Transverse section of silkworm at the final stage infected by virus; positive signals occur in all tissues (bar is 90 μm).

*Bm*DNV-like is a single chain DNA virus with a genome of about 6 500 nt. It reproduced in nuclei of the infected tissues of diseased silkworm, resulting in nuclei being inflated, with typical features of Densovirinae. The size of the inflated nucleus caused by *Bm*DNV-like was 2.6 times as that of normal nucleus, which was far more larger than that caused by *Bm*DNV-Ⅰ (1.5 times), but close to that caused by *Bm*DNV-Ⅱ, which was 2.5 times as that of normal nucleus (Watanabe and Kurihara, 1988). Under electron microscope, the virions seen by *Bm*DNV-Ⅰ are not found in the viral matrix of *Bm*DNV-like. Regarding the arrangement of virus particles, linear arrangement like *Bm*DNV-Ⅱ was also not found. The same as the other two *Bm*DNVs (*Bm*DNV-Ⅰ and *Bm*DNV-Ⅱ), *Bm*DNV-like virus first infected columnar cells in midgut of silkworm, multiplied and entered into intestines antrum after wrecking the nucleus. But all the cells of midgut including goblet cells were also infected by *Bm*DNV-like, and the *Bm*DNV-like can cause systemic infection of silkworm at the final stage of infection. However, *Bm*DNV-Ⅰ and *Bm*DNV-Ⅱ only infected columnar cells of midgut, it could not infect other tissues. In addition, *Bm*DNV-like has no serology kinship with *Bm*DNV-Ⅰ and *Bm*DNV-Ⅱ. Therefore, *Bm*DNV-like differs greatly from DNVs already found in silkworm. Based on the rule of virus classification, it may be a new type of *Bm*DNV.

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一种家蚕类浓核病毒的组织病理学特征

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摘要： 本文从家蚕病蚕中分离到一种家蚕类浓核病毒（*Bm*DNV-Like），对它的组织病理学研究表明：该病毒首先寄生家蚕中肠柱状细胞，继而引起其细胞核的膨大和破裂；组织原位杂交结果表明该病毒既能在家蚕中肠柱状细胞中增殖，也能在中肠的杯形细胞中增殖，甚至在感染后期能在家蚕幼虫的大部分组织细胞中感染和增殖。

关键词： 浓核病毒；家蚕；组织病理学特征
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